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Cowpea mosaic virus RNA-1 acts as an amplicon whose effects can be counteracted by a RNA-2-encoded suppressor of silencing

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Abstract

Lines of *Nicotiana benthamiana* transgenic for full-length copies of both *Cowpea mosaic virus* (CPMV) genomic RNAs, either singly or together, have been produced. Plants transgenic for both RNAs developed symptoms characteristic of a CPMV infection. When plants transgenic for RNA-1 were agro-inoculated with RNA-2, no infection developed and the plants were also resistant to challenge with CPMV. By contrast, plants transgenic for RNA-2 became infected when agro-inoculated with RNA-1 and were fully susceptible to CPMV infection. The resistance of RNA-1 transgenic plants was shown to be related to the ability of RNA-1 to self-replicate and act as an amplicon. The ability of transgenically expressed RNA-2 to counteract the amplicon effect suggested that it encodes a suppressor of posttranscriptional gene silencing (PTGS). By examining the ability of portions of RNA-2 to reverse PTGS in *N. benthamiana*, we have identified the small (S) coat protein as the CPMV RNA-2-encoded suppressor of PTGS.

Keywords: *Cowpea mosaic virus*; RNA-1 transgenic plants; RNA-2 transgenic plants; Resistance; Complementation; Amplicon; RNA-2-encoded suppressor of posttranscriptional gene silencing; Small (S) coat protein

Introduction

Cowpea mosaic virus (CPMV) is a bipartite RNA plant virus. Both genomic RNAs are expressed via the production and subsequent processing of polyproteins. RNA-1 encodes proteins involved in RNA replication although RNA-2 encodes a protein that allows the virus to move from cell to cell (movement protein; MP) and the two viral coat proteins, Large (L) and Small (S). Although both RNAs are required for a productive infection of whole plants, RNA-1 can replicate independently in protoplasts (Goldbach et al., 1980). By contrast, RNA-2 is completely dependent on the RNA-1-encoded proteins for its replication.

Although portions of the CPMV genome have previously been introduced into plants as transgenes (e.g., Sijen et al., 1995), there are no reports of the production of transgenic plants containing full-length, replication-competent versions of the genomic RNAs. As part of our studies on the use of CPMV as an expression system, we have transformed *Nicotiana benthamiana*, a systemic host for CPMV, with cDNA copies of both genomic RNAs either singly or together. Plants transgenic for both genomic segments developed symptoms characteristic of a CPMV infection although those transformed with the individual genome segments were phenotypically normal. Plants transgenic for a full-length, replication-competent version of RNA-1 are resistant to virus infection as a result of the transgenically expressed RNA-1 acting as an amplicon (Angell and Baulcombe, 1997). We further show that RNA-2 encodes a suppressor of gene silencing that can counteract the amplicon effect and the protein responsible is the Small (S) coat protein.

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Results

N. benthamiana plants transgenic for CPMV RNA-1 and RNA-2 produce levels of virus similar to those found in CPMV-infected plants

N. benthamiana plants were cotransformed with plasmids pBinPS1NT and pBinPS2NT (Fig. 1). These plasmids contain full-length cDNA copies of CPMV RNA-1 and RNA-2, respectively, inserted between a CaMV 35S promoter and a *nos* terminator and have been used previously for agro-infection (Liu and Lomonossoff, 2002). PCR analysis of genomic DNA identified a plant, termed 12NT-7, which was transformed with both transgenes. On transfer to soil, this plant developed symptoms characteristic of a wild-type CPMV infection (Figs. 2A, B). To confirm the presence of infectious virus particles in this plant, sap samples were inoculated onto *Phaseolus vulgaris* cv "Canadian wonder", a local lesion host for CPMV, at various dilutions. The results demonstrated that the plant transgenic for RNA-1 and RNA-2 produced levels of infectious virions indistinguishable from a natural CPMV infection (data not shown).

To examine whether the infection derived from the multiplication of transgene-derived mRNA in line 12NT-7 had the same cytopathological effects as a natural CPMV infection, tissue sections were examined by electron microscopy. Cells from symptomatic tissue from both 12NT-7 transgenic and CPMV-infected *N. benthamiana* plants contained large numbers of icosahedral particles (Fig. 2C) that were shown to be CPMV particles by immunogold-labeling with anti-CPMV antibodies (data not shown). It was particularly noticeable that modified plasmodesmata containing virus particles within tubules could be seen in samples from both the transgenic and CPMV-infected plants (Fig. 2C). To determine whether the "infected" phenotype observed with line 12NT-7 was heritable, the plant was self-fertilized and the seeds collected. When the F1 generation was analyzed, 30 out of 42 plants recovered from seed showed symptoms characteristic of a CPMV infection. This ratio suggests that the original plant was heterozygous for both the RNA-1 and RNA-2 transgenes. Symptomatic plants could also be recovered from the F2 generations derived from these plants. Separate experiments using lines of plants transgenic for a version of RNA-2 carrying GFP have shown that the simultaneous presence of RNA-1 and RNA-2 transgenes invariably leads to a productive infection (Cañizares, M.C. Tsakiris, E., Liu, L., Perrin, Y. and Lomonossoff, G.P., unpublished observations).

Complementation and resistance in plants transgenic for individual CPMV genome segments

Plasmids pBinPS1NT and pBinPS2NT (Fig. 1) were used individually to produce lines of *N. benthamiana* transgenic for either RNA-1 or RNA-2. Lines transgenic for RNA-1 were given the designation 1NT-*n* and those for RNA-2 were designated 2NT-*n*, where *n* is the number of a given line. In

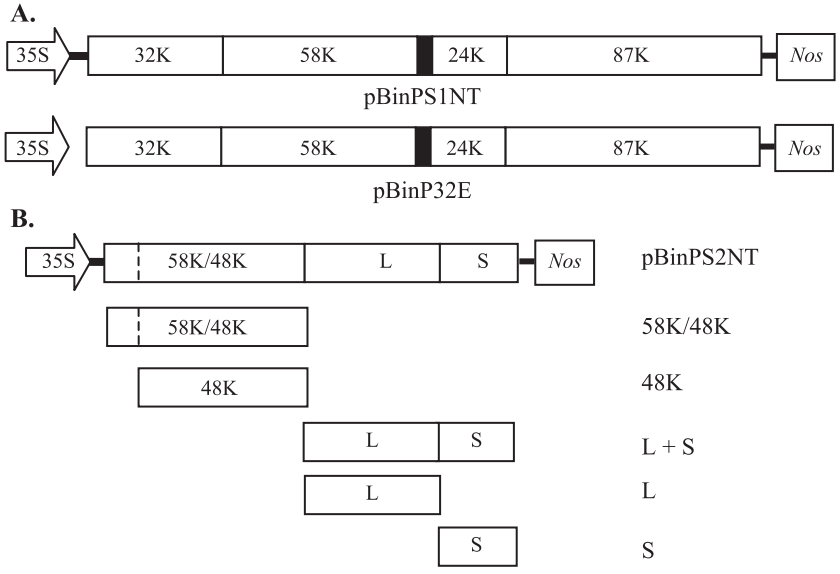

Fig. 1. CPMV constructs used for transformation and agro-inoculation. (A) Structure of the CPMV-specific portions of RNA-1-based plasmids pBinPS1NT and pBinP32E. These plasmids contain full-length cDNA copies of CPMV RNA-1 and a version of RNA-1 lacking the 5′ UTR, respectively, between the 35S promoter and *nos* terminator in pBINPLUS. The ORF encoding the 200K protein is shown as an open box with sites of cleavage in the polyproteins indicated by vertical lines, together with the designation of the cleavage products (32K, etc.). The region encoding the VPg is shown as a black rectangle. (B) Structure of CPMV-specific portions of RNA-2-based plasmids. pBinS2NT contains a full-length of CPMV RNA-2 between the 35S promoter and *nos* terminator in pBINPLUS. The ORF encoding the 105 and 95K proteins is shown as open box with the site for the initiation of translation of the 95K protein indicated by a dotted line. The sites of cleavage in the polyproteins are indicated by vertical lines, together with the designation of the cleavage products (58K, etc.). 58K/48K, 48K, L, S, and L + S show the portions of RNA-2 inserted into the 35S- and PVX-based vectors.

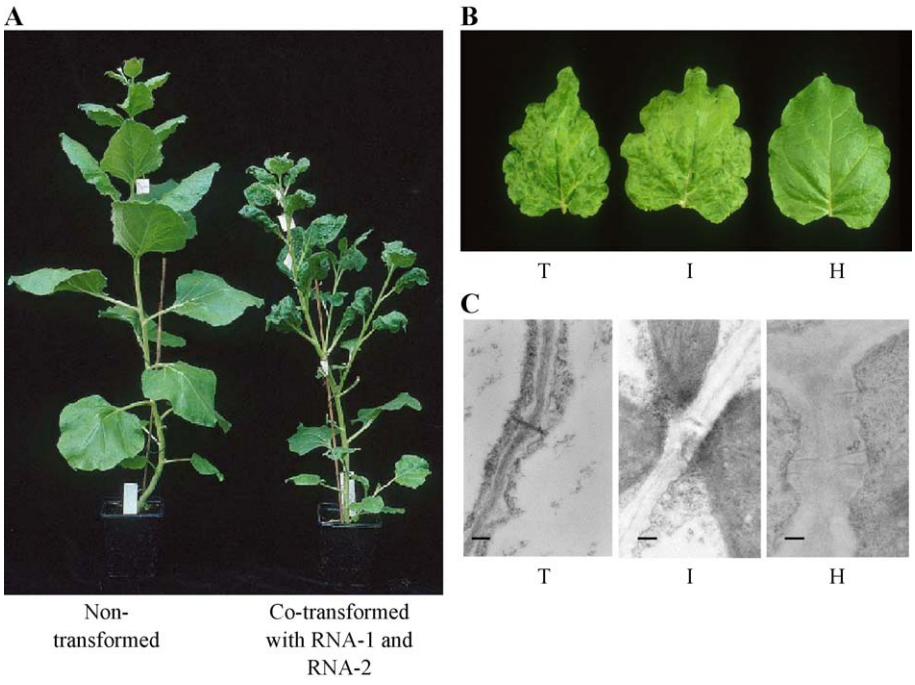

Fig. 2. Effect of the simultaneous presence of RNA-1 and RNA-2 transgenes on the appearance of *N. benthamiana* plants. (A) Comparison of appearance of *N. benthamiana* plant transgenic for CPMV RNA-1 and RNA-2 (right) with that of a nontransformed plant of the same age (left). (B) Comparison of symptoms on upper leaves of a plant transgenic for RNA-1 and RNA-2 (T) with those of a plant systemically infected with CPMV (I) and a healthy control (H). (C) Electron micrographs of thin sections of leaf tissue from a *N. benthamiana* plant transgenic for CPMV RNA-1 and RNA-2 (T), a plant infected with CPMV (I) or a healthy plant (H). Scale bar = 200 nm.

addition, plasmid pBinP32E (Fig. 1A) containing a version of RNA-1 lacking the 5′ UTR was also used to transform plants. Because the 5′ UTR is essential for RNA replication, this deleted version of RNA-1, while retaining the entire RNA-1 open reading frame (ORF), will be replication deficient. None of the plants transgenic for the individual genome segments displayed any symptoms of virus infection.

To determine whether exogenously supplied genome segments could complement integrated copies of the other segment, *N. benthamiana* plants transgenic for either full-

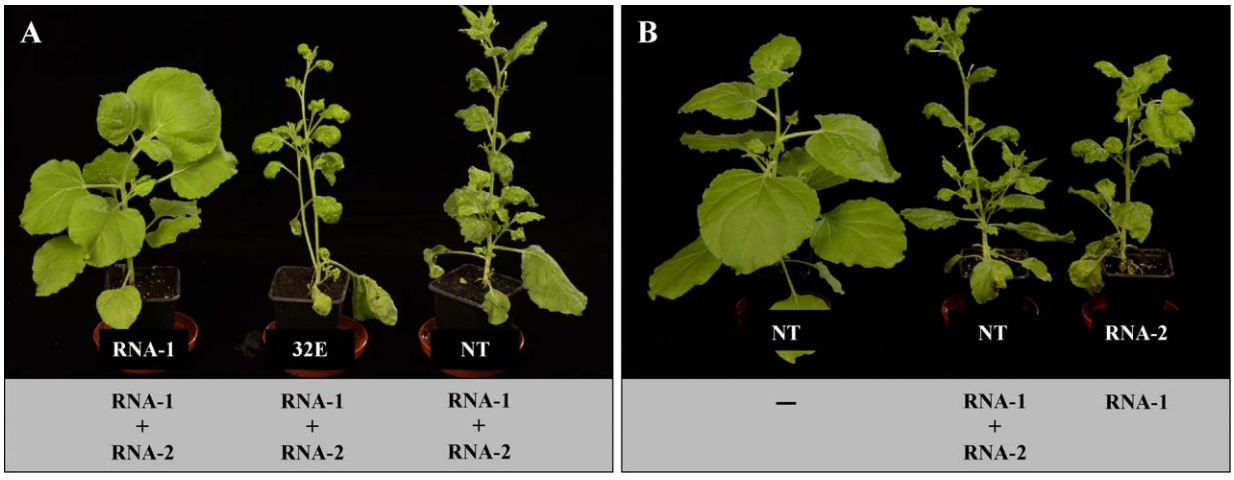

Fig. 3. Symptoms on *N. benthamiana* plants agro-inoculated with CPMV RNA constructs. In each case, the transgene status of the plant is indicated by the pot while the inoculum used is indicated below each plant. (A) Effect of challenge with CPMV RNA-1 and -2 on *N. benthamiana* plants transgenic for either full-length RNA-1 (RNA-1; left), construct 1NT-32E lacking the 5′ UTR of RNA-1 (32E; center), or a nontransgenic plant (NT; right). (B) Symptoms on a plant transgenic for CPMV RNA-2 agro-inoculated with RNA-1 (right) compared with those of NT plant agro-inoculated with both RNA-1 and RNA-2 (center) and non-inoculated NT plant (left). The plants were photographed 23 days post-inoculation.

length RNA-1, replication-deficient RNA-1 or RNA-2 were agro-inoculated with the complementary genome segment. As controls, plants from each line were also agro-inoculated with both genome segments. None of plants from the three lines transgenic for full-length RNA-1 (1NT-1, -2, and -3) developed symptoms when agro-inoculated with either CPMV RNA-2 alone or with both RNA-1 and -2 even when left for several weeks (Table 1; Fig. 3A). In addition, plants from a homozygous F2 line, 1NT-11F2, derived from line 1NT-1, also failed to develop symptoms (Table 1; Fig. 3A). Thus, the RNA-1 transgene in these plants not only failed to complement exogenously supplied RNA-2 but also apparently conferred resistance to CPMV. This resistance was not confined to the application of CPMV by agro-inoculation, because similar results were obtained when the RNA-1 transgenic plants were mechanically inoculated with CPMV.

The apparent resistance of plants transgenic for RNA-1 was confirmed by a local lesion assay. When undiluted sap from the upper leaves of 1NT-11F2 plants that had been agro-inoculated with RNA-1 and RNA-2 was used to inoculate *P. vulgaris*, no lesions developed. By contrast, a 1:100 dilution of sap from equivalent tissue from nontransgenic plants agro-inoculated with RNA-1 and RNA-2 gave several hundred lesions per leaf. Furthermore, the sap from the 1NT-11F2 plants could not initiate an infection on the systemic host, *Vigna unguiculata* (cowpea). Western blot analysis of protein extracts using a CPMV-specific serum also failed to reveal the presence of the viral coat proteins in the upper leaves of 1NT-11F2 plants agro-inoculated with RNA-1 and RNA-2 although the coat proteins were easily detectable in extracts of infected nontransgenic plants (data not shown).

Failure of exogenously supplied RNA-2 to cause an infection was also observed in plants transgenic for the replication-deficient version of RNA-1 (lines 1NT-32E-3-1 and -32E-9-2; Table 1). However, in this case, the plants were fully susceptible to challenge with CPMV in two separate experiments (Table 1; Fig. 3A), developing symptoms within 1 week post-inoculation. A local lesion assay showed that sap from agro-inoculated 1NT32E plants contained similar levels of virus to that found in CPMV-infected nontransgenic plants.

Table 1
Complementation with exogenously applied genome segments

Transgenic line	No. of plants infected[a]/No. of plants inoculated			
	With complementary segment[b]		With both segments[c]	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
1NT-1	0/16		0/20	
1NT-2	0/20		0/20	
1NT-3	0/18		0/20	
1NT-11F2	0/3	0/8	0/5	0/4
1NT-32E-3-1	0/10	0/3	10/10	5/5
1NT-32E-9-2	0/8	0/3	8/8	5/5
2NT-5	20/20	8/8	20/20	4/4
2NT-7	12/20	8/8	20/20	4/4
2NT-8	20/20	8/8	n.d.[d]	4/4

[a] As judged by the appearance of characteristic symptoms.
[b] In the case of plants transgenic for RNA-1 (1NT), RNA-2 was agro-infiltrated, whilst in the case of RNA-2 transgenics (2NT), RNA-1 was agro-infiltrated.
[c] Plants were agro-infiltrated with both RNA-1 and RNA-2.
[d] n.d. = not determined.

In contrast to results obtained with the plants transgenic for RNA-1, exogenously supplied RNA-1 was able to cause an infection when inoculated onto RNA-2 transgenic plants. A total of 76 out of 84 plants from three lines transgenic for RNA-2 (2NT-5, -7, and -8) developed symptoms indistinguishable from a wild-type infection when agro-inoculated with CPMV RNA-1 (Table 1; Fig. 3B). In addition, all the RNA-2 transgenic plants developed symptoms when agro-inoculated with both RNA-1 and RNA-2 (Table 1). The symptoms were identical in their severity and duration to those found on nontransgenic plants inoculated with CPMV and persisted for at least 65 days post-inoculation with no sign of recovery.

Complementation between RNA-1 and -2 can be achieved by crossing

To determine whether an infected phenotype could be achieved by crossing plants transgenic for the individual genome segments, various RNA-1- and RNA-2-transgenic lines were crossed. When line 1NT-1 was crossed to lines 2NT-7, -8, or -25, 1/22, 1/8, and 3/5 plants, respectively, recovered after germinating the seed showed the infected phenotype. When line 1NT-2 was crossed with 2NT-7, 1/20 of progeny plants had the infected phenotype. The relatively low proportion of plants developing symptoms of infection is probably due to a combination of the fact that the crosses were performed using primary transformants, which were not homozygous, as parents and the likelihood that the presence of both transgenes, with the concomitant symptoms of virus infection, results in some degree of embryo lethality. These results show that an infected phenotype can be achieved with plants transgenic for RNA-1 if a copy of RNA-2 is introduced by crossing rather than exogenous application. In addition, the results confirm that the copies of RNA-1 contained in lines 1NT-1 and -2 are fully replication competent.

Transgene-derived, full-length RNA-1 can replicate

To confirm that transgene-derived mRNA from plants transgenic for the full-length version of RNA-1 is capable of self-replication, RNA was extracted from leaves of a 1NT-11F2 and a 1NT32E-3-1 plant and subjected to Northern blot analysis using strand-specific probes. In both cases, apparently full-length positive-sense RNA molecules could be detected (Fig. 4A), with noticeably higher amounts being found in the sample from the plant transgenic for full-length RNA-1. The positive-sense RNA detected in the 1NT-32E sample represents the product of nuclear transcription in the

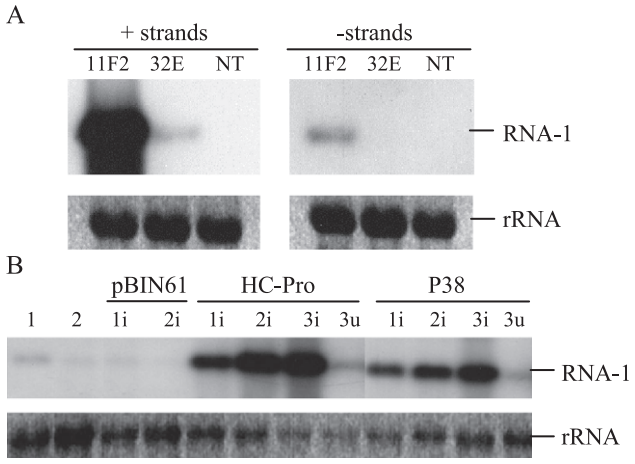

Fig. 4. Analysis of RNA-1 sequences present in total RNA isolated from leaves of *N. benthamiana* plants. (A) Upper panels: Northern blots of RNA extracted from transgenic lines 1NT-11F2, -32E, or nontransgenic plants probed with sequences specific for either positive (+ strands)- or negative (− strands)-sense RNA-1. The position of full-length RNA-1 is indicated. (B) Upper panel: Northern blot showing levels of RNA-1 CPMV (−) strands RNA in samples isolated from plants that were either not agro-infiltrated (1, 2) or agro-infiltrated with either the empty pBIN61 vector (1i, 2i), 35S-HC-Pro (1i, 2i, 3i), or 35S-P38 (1i, 2i, 3i). In addition, RNA isolated from an upper, non-infiltrated leaf (3u) of plants agro-infiltrated on a lower leaf with each of the constructs was analyzed. The RNA was extracted from leaves 6 days post-infiltration. Lower panels of A and B show ethidium bromide stained gels of the same samples used for Northern analysis to assess the levels of ribosomal RNA (rRNA) as a loading control.

absence of replication. Although full-length negative-strand RNA could be detected in the sample from the 1NT-11F2 plant, no equivalent signal could be detected in the 1NT-32E-3-1 sample. This provides direct evidence that transgene-derived full-length RNA-1 is capable of self-replication and confirms that replication is abolished by deletion of the 5′ UTR.

CPMV RNA-1 expressed from a transgene acts as an amplicon

The most probable reason for the consistent anti-CPMV resistance found in plants transgenic for full-length RNA-1 is that its ability to replicate efficiently triggers posttranscriptional gene silencing (PTGS). Thus, the integrated copy of RNA-1 would act as an amplicon in a manner analogous to that found with an integrated copy of the *Potato Virus X* (PVX) genome (Angell and Baulcombe, 1997). If this is the case, infiltration of leaves of plants transgenic for RNA-1 with known suppressors of PTGS should be able to reverse the silencing, leading to an increased accumulation of RNA-1-specific sequences. To investigate this, leaves of three different 1NT-11F2 plants were agro-infiltrated with pBIN61-based constructs containing sequences encoding either the helper component-proteinase (HC-Pro) from *Potato virus Y* (PVY) or the coat protein (P38) of *Turnip crinkle virus* (TCV), both of which are well-characterized suppressors of PTGS. RNA was isolated and probed for the presence of RNA-1-specific negative strand RNA. As controls, RNA was extracted from leaves that were either not agro-infiltrated, agro-infiltrated with bacteria containing the empty pBIN61 vector, or from the upper (non-infiltrated) leaves of plants that had been agro-infiltrated with either of the two suppressors. The results show that there is a substantial increase in the levels of RNA-1 negative strands after agro-infiltration with either suppressor but not with the empty pBIN61 vector (Fig. 4B). This effect was restricted to the leaves that were actually infiltrated, no increase in RNA-1 negative strands being seen in the upper leaves of these plants.

Confirmation that CPMV RNA-2 encodes a suppressor of PTGS

The observation that the amplicon effect of transgenically expressed RNA-1 can be reversed not only by known suppressors of PTGS (see above) but also by the simultaneous presence of a RNA-2 transgene, introduced either by cotransformation or crossing, suggests that RNA-2 may encode a suppressor of PTGS. The notion that CPMV encodes a suppressor is supported by the previous observation that CPMV infection leads to partial restoration of GFP expression in previously silenced plants (Voinnet et al., 1999) and by the fact that plants transgenic for RNA-2 display no resistance to subsequent challenge with CPMV.

To test for the existence of a CPMV-encoded suppressor of PTGS, GFP leaf patch tests were conducted using either nontransgenic *N. benthamiana* or *N. benthamiana* transgenic for 35S-GFP (line 16c) as previously described by Voinnet et al. (1998). In these assays, a 35S-GFP construct was agro-infiltrated into *N. benthamiana* leaves. Two days post-inoculation high-level GFP expression could be observed under UV light in the infiltrated patches of both nontransgenic (NT) and 16c leaves. By 6–7 days post-inoculation, the GFP patch on the NT leaves had faded (Fig. 5A, panels marked −), a phenomenon attributed to PTGS of the transgene (Johansen and Carrington, 2001). On 16c leaves, a red line at the periphery of the inoculated patch could be observed, corresponding to silencing of both the endogenous and exogenous GFP transgene. When 35S-HC-Pro was co-inoculated with 35S-GFP, the GFP expression levels in the inoculated patch remained high after 6–7 days in both nontransgenic and 16c plants and no red line of silencing was observed in 16c leaves (Fig. 5A, panels marked 35S-HC-Pro).

The existence of a CPMV-encoded suppressor was examined by the above GFP patch test by infiltrating pBinPS1NT and pBinPS2NT (35S-RNA-1 and 35S-RNA-2, respectively), either separately or together, at the same time as 35S-GFP. Although the presence of either RNA-1 or RNA-2 alone did not suppress the PTGS of GFP in either NT or 16c plants (Fig. 5A, panels 35S-RNA-1 and 35S-RNA-2), the simultaneous presence of both genome segments resulted in the persistence of GFP expression in the

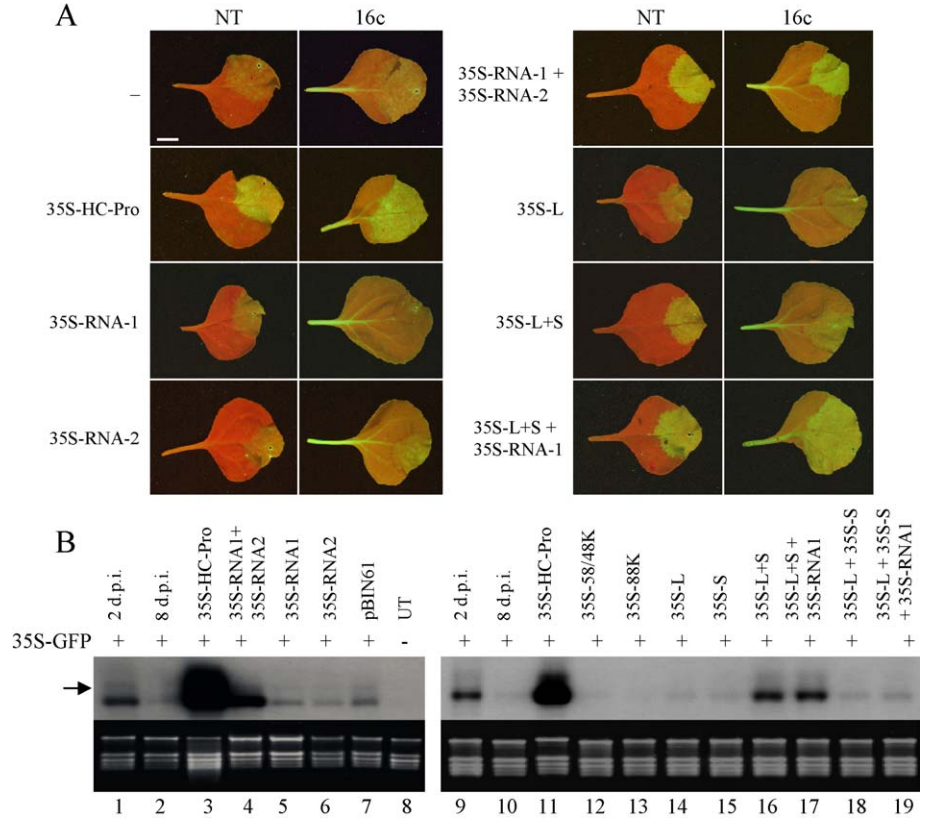

Fig. 5. Identification of the CPMV suppressor of PTGS by GFP patch tests. (A) Nontransgenic (NT) and 16c *N. benthamiana* leaves viewed under UV light at 5 days post agro-inoculation with 35S-GFP. The leaves were either inoculated with 35S-GFP alone (panels marked −) or co-inoculated with 35S-GFP and the construct indicated beside each panel. Scale bar = 10 mm. (B) Northern blot analysis of RNA extracted from infiltrated leaf patches of nontransgenic *N. benthamiana*. RNA was isolated from leaves 8 days post-infiltration except for the samples in lanes 1 and 9, which were taken 2 days post-infiltration to assess the levels of GFP expression before silencing was induced. The upper panels show Northern blots of GFP mRNA accumulation using a GFP-specific probe, whilst the lower panels show the ethidium bromide-stained RNA gels before transfer as a loading control. In all cases except lane 8 (untreated; UT), leaves were inoculated with 35S-GFP and the 35S promoter-driven expression cassettes indicated above the lane. The position of GFP mRNA is indicated.

nontransgenic *N. benthamiana* and the abolition of the red line of silencing in the 16c plants (Fig. 5A, panel 35S-RNA-1 + 35S-RNA-2). Northern blot analysis of RNA extracted from the infiltrated leaves using a GFP-specific probe confirmed that levels of GFP mRNA declined after infiltration (compare lanes 1 and 2 in Fig. 5B), and this decline could be reversed by co-infiltration with 35S-HC-Pro. Increased levels of GFP-specific mRNA could also be seen in samples from leaves co-infiltrated with both 35S-RNA-1 and 35S-RNA-2 together, but not when either construct was infiltrated separately (Fig. 5B, lanes 4–6). However, the enhancement of GFP mRNA levels was considerably less dramatic than that seen with 35S-HC-Pro, suggesting that the CPMV-encoded suppressor is relatively weak. No increased GFP mRNA levels could be detected in samples from leaves infiltrated with an empty 35S-containing vector (lane 7) and no GFP mRNA was detected in leaves that were not infiltrated at all (lane 8). Similar results were obtained when RNA extracted from 16c leaves was analyzed (data not shown). The above results confirm the previous data indicating that CPMV encodes a suppressor of PTGS, and also provide indirect evidence that this suppressor is encoded by RNA-2. Because RNA-1 encodes the viral 24K proteinase, the RNA-1-encoded polyprotein can be processed to the mature products in the absence of RNA-2. Thus, any suppressor encoded by RNA-1 should be expressed equally well in both the presence and absence of RNA-2. This is clearly not the case. By contrast, the RNA-2-encoded polyprotein is completely dependent on the RNA-1-encoded proteinase for its processing, and in the absence of RNA-1, no mature products will be formed. Thus, if the suppressor is encoded by RNA-2, its activity would not be evident in the absence of RNA-1 because, in the absence of processing, the RNA-2-encoded polyproteins are highly unstable (Wellink et al., 1987).

Identification of the RNA-2-encoded suppressor of PTGS

To identify which CPMV RNA-2-encoded protein is the suppressor of PTGS, portions of RNA-2 encoding the individual mature proteins (Fig. 1B) were inserted into pBIN61 to allow 35S-driven expression in plant cells. Construct 35S-58K/48K was designed to express the overlapping 58K (unknown function) and 48K (movement)

proteins, whilst constructs 35S-48K, 35S-L, and 35S-S were designed to individually express the 48K movement protein and the Large (L) and Small (S) coat proteins, respectively. Because an intermediate processing product, consisting of an L + S fusion protein, can also be found in infected plant tissue (Wellink et al., 1987), a construct expressing this, 35S-L + S (Fig. 1B), was also produced.

When the constructs encoding the RNA-2 proteins (35S-48K, -58K/48K-L, or -S) were examined in the GFP patch test in both nontransgenic and 16c plants, the levels of GFP fluorescence were similar to those achieved with expression of the GFP plasmid alone (only the result with 35S-L is shown in Fig. 5A, panel 35S-L). In all these instances, no enhanced levels of GFP-specific mRNA could be detected (Fig. 5B, lanes 12–15). By contrast, infiltration with 35S-L + S resulted in elevated GFP expression levels in the inoculated patches of both nontransgenic and 16c leaves, indicating the presence of suppressor activity (compare Fig. 5B, lanes 10 and 16). However, on 16c leaves, the ring of transgene silencing at the patch periphery was still initiated [Fig. 5A, panel 35S-L + S. The enhanced levels of GFP fluorescence correlated with enhanced levels of GFP mRNA (Fig. 5B, lane 16)], though the degree of GFP mRNA enhancement was noticeably less than that achieved with 35S-HC-Pro (Fig. 5B), consistent with the idea that CPMV encodes a weak suppressor of silencing. This result also suggests that the L + S fusion protein, rather than either individual coat protein, possesses the suppressor activity, but this is insufficient to prevent the triggering of PTGS in the 35S-GFP transgenic line 16c. To clarify the situation, ELISA analysis was carried out on extracts from the patches infiltrated with 35S-L, 35S-S, and 35S-L + S using an antibody raised against CPMV virions. The results showed that although the L and L + S proteins were expressed to detectable levels in the patches, the S coat protein was not. This lack of expression of the S coat protein in leaves infiltrated with 35S-S was confirmed by Western blot analysis (data not shown). The failure to achieve detectable expression of the S coat protein in infiltrated patches was not due to errors in the construction of 35S-S and could not be overcome by remaking it. Our failure to achieve expression of the S coat protein leaves open the possibility that the suppressor of silencing is actually the S protein portion of the L + S fusion, rather than specifically the fusion protein itself.

To determine whether covalent attachment of the L and S subunits in the protein expressed from L + S construct is necessary to achieve suppression, we tested the effect of protein processing on its suppressor activity. To this end, 35S-L + S was infiltrated into *N. benthamiana* leaves in the presence of 35S-RNA-1. Cleavage of the L + S protein into the mature L and S subunits by the RNA-1-encoded proteinase was confirmed by Western blot analysis, using anti-CPMV antibodies, of protein samples from the inoculated patches. The co-inoculated nontransgenic leaves showed high levels of persistent GFP fluorescence (Fig. 5A, panel 35S-L + S + 35S-RNA-1), and enhanced levels of GFP mRNA could be detected in RNA extracted from the leaves (Fig. 5B, lane 17). In addition, the red front of silencing in inoculated 16c leaves was abolished (Fig. 5A). By contrast, increased levels of GFP mRNA were not found in extracts co-infiltrated with 35S-L and 35S-S in the presence or absence of RNA-1, a result consistent with our previous failure to detect the synthesis of the S coat protein from construct 35S-S. These results show that covalent linkage of the L and S subunits is not required to obtain suppression of PTGS and, if anything, the abolition of the ring of silencing in the 16c plants suggests that processing at the L–S junction actually enhances suppressor activity. Taken in conjunction with the data showing that the L protein expressed from the 35S-L construct did not provide the suppressor function, these experiments point to the suppressor of PTGS being associated with the S protein subunit, though apparently this may only be manifested when it is noncovalently associated with the L subunit.

Confirmation that the CPMV S coat protein is a suppressor of PTGS

In an attempt to confirm that the CPMV-encoded suppressor activity is associated with the S coat protein, the regions of RNA-2 encoding the individual proteins were cloned into a PVX-based vector downstream of a duplicated subgenomic promoter. It was hoped that this approach might permit sufficient levels of the S protein to accumulate so that its potential involvement in suppression of PTGS could be assessed. The constructs were designed to express the overlapping 58K and 48K proteins (PVX::58K/48K), the 48K MP alone (PVX::48K), and the L (PVX::L) and S (PVX::S) coat proteins (Fig. 1B).

To assay the suppressor activity of the CPMV proteins, 16c plants were silenced by agro-infiltration with a 35S-GFP-RNAi construct. As soon as complete silencing of GFP was observed throughout the whole plant, as shown by total loss of GFP fluorescence under UV light (Fig. 6A, panel 1), the plants were agro-inoculated with appropriate constructs. As a control, the GFP-silenced 16c plants were inoculated with a combination of pBinPS1NT and pBinPS2NT to give a CPMV infection. This resulted in the suppression of GFP silencing in a vein-centric pattern (Fig. 6A, panel 2), a result consistent with that reported previously by Voinnet et al. (1999). Scanning laser confocal microscopy revealed that although GFP levels were higher in vascular cells, GFP could also be detected in some cells of the leaf lamina (Fig. 6A, panel 3). Thus, the CPMV suppressor activity is vein-centric but not vein-restricted. The ability of a CPMV infection to partially reverse silencing was confirmed by Northern blot analysis of GFP mRNA in leaf tissue (Fig. 6B, lanes 1–4). Though the ability of a CPMV infection to increase the levels of GFP mRNA was limited (compare lanes 3 and 4), it is consistent with the results reported by

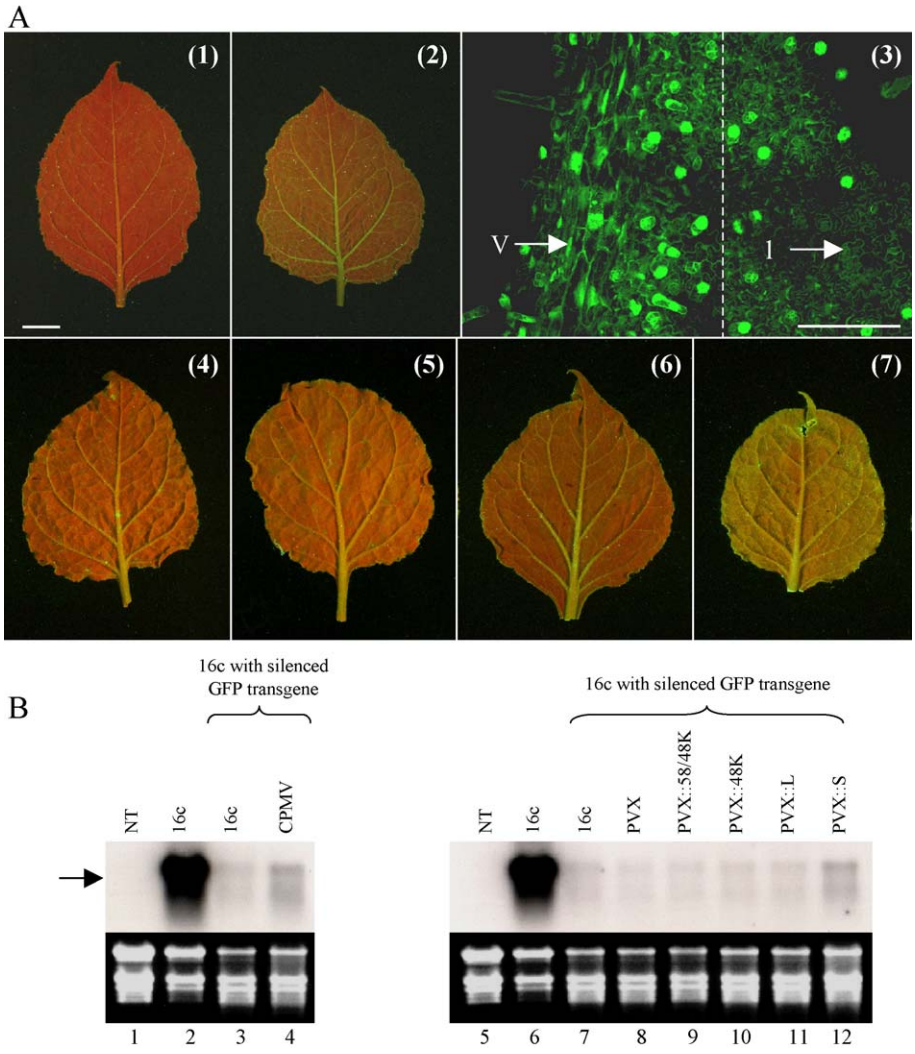

Fig. 6. Suppression of PTGS in *N. benthamiana* leaves containing a silenced GFP transgene. (A) Abaxial surfaces of leaves from silenced 16c plants viewed under UV light. Panel 1: Systemic leaf from GFP-silenced plant. Scale bar = 10 mm; Panel 2: systemic silenced leaf from CPMV-infected plant; Panel 3: Adjoining confocal micrographs of systemic leaf from CPMV-infected plant, viewed at 488 nm. V = vein, l = lamina. Scale bar = 5 mm; Panel 4: Systemic silenced leaf from PVX-infected plant; Panel 5: Systemic silenced leaf from PVX::L-infected plant; Panels 6 and 7: Systemic silenced leaves from two different PVX::S-infected plants showing either vein-centric (6) or overall (7) fluorescence. (B) Northern blot analysis of RNA extracted from silenced 16c leaves infected with CPMV or PVX constructs containing portions of CPMV RNA-2. The upper panels show Northern blots of GFP mRNA accumulation using a GFP-specific probe whilst the lower panels show the ethidium bromide-stained RNA gels before transfer as a loading control. Lanes 1 and 5: Extracts from NT plants; Lanes 2 and 6; extracts from nonsilenced 16c plants; Lanes 3 and 7: extracts from silenced 16c plants; Lane 4: extract from silenced leaf infected with CPMV; Lanes 8–12: extracts from silenced leaves infected with PVX (8), PVX::58K/48K (9), PVX::48K (10), PVX::L (11), and PVX::S (12). The position of GFP mRNA is indicated. The lower panel show the ethidium bromide-stained RNA gels before transfer as a loading control.

Voinnet et al. (1999), which indicated that CPMV encodes a weak suppressor of PTGS.

GFP-silenced 16c plants agro-inoculated with the various PVX-based constructs were examined under ultraviolet light once strong symptoms of PVX infection were observed. Expression of the CPMV S protein could be detected by ELISA in leaves infected with PVX::S (data not shown). Infection with the empty PVX vector or PVX::L did not result in suppression of GFP silencing (Fig. 6A, panels 4 and 5) and similar negative results were also obtained with PVX::58/48K and PVX::48K (data not shown). Although occasionally some regions of apparent fluorescence were observed on leaves infected with these constructs (e.g., Fig. 6A, panel 4), these were correlated with necrosis due to the PVX infection rather than being the result of genuine GFP fluorescence. By contrast, infection with PVX::S resulted in a clear increase in GFP expression (Fig. 6A, panels 6 and 7). In some plants, this was observed in a vein-centric manner consistent with the pattern of suppression observed during a CPMV infection (compare Fig. 6A, panels 2 and 6), although in others, suppression of GFP silencing was observed as a general elevation of GFP levels in the leaf (panel 7). To confirm that the apparent increase in fluorescence was associated with enhanced levels of GFP-specific

mRNA, RNA was extracted from leaves infected with each of the PVX constructs and subjected to Northern blot analysis with a GFP-specific probe (Fig. 6B, lanes 8–12). Though the effect is somewhat weak, the only sample showing enhanced levels of GFP mRNA was that from tissue infected with PVX::S (Fig. 6B, lane 12). Densitometry of the Northern blot indicated that the level of enhancement was approximately 3.5-fold over the samples not showing suppression. This was similar to that found during a CPMV infection (Fig. 6B, compare lanes 3 and 4). This is consistent with the S protein being a weak suppressor of PTGS in this assay. Overall, these results confirm the indication from the GFP patch test that the S coat protein is the CPMV-encoded suppressor of PTGS.

Discussion

We have shown that transformation of *N. benthamiana* plants with both genome segments of CPMV results in a productive virus infection. Although infection can be achieved by inoculating plants transgenic for CPMV RNA-2 with RNA-1, no infection results when RNA-1 transgenic plants are inoculated with RNA-2. In addition, RNA-1 transgenic plants showed consistent resistance to challenge with CPMV while the RNA-2 transgenic plants were fully susceptible. Because RNA-1 can self-replicate, the resistance observed can be explained by RNA-1 acting as an amplicon, thereby inducing consistent PTGS as previously found with PVX (Angell and Baulcombe, 1997). However, the situation is distinct from that previously reported for *Tobacco mosaic virus*, where integration of a full-length copy of the genomic RNA resulted in a productive infection (Yamaya et al., 1988). The only previous report of resistance in plants transgenic for the genome segments of a multipartite virus concerned *Brome mosaic virus* (BMV; Kaido et al., 1995). It was found that plants transformed with all three BMV genomic segments produced only low levels of infectious virus, in contrast to the productive infection found in our CPMV RNA-1 and -2 transgenic lines. Protoplasts isolated from plants containing the entire BMV genome were also resistant to superinfection with BMV. Resistance was also observed in protoplasts isolated from plants transgenic for BMV RNA-1 and RNA-2 alone, but not in protoplasts isolated from plants transgenic for copies of RNA-1 and RNA-3 or RNA-2 and RNA-3. Unfortunately, no attempt was made to assess the resistance of intact plants (rather than protoplasts) making a detailed comparison with the properties of our CPMV RNA-1 transgenic lines impossible.

The association between the ability of RNA-1 to self-replicate and induce consistent resistance in transgenic plants was confirmed by creating lines of plants transgenic for a replication-defective version of RNA-1 with a deletion in the 5′ UTR (1NT-32E). Plants transgenic for this construct were unable to complement the replication of exogenously supplied RNA-2 and were not resistant to CPMV infection. The lack of complementation was somewhat surprising as it had previously been shown that cowpea protoplasts transiently expressing the RNA-1 200K ORF from a virtually identical RNA-1 construct (pMB200) lacking the 5′ UTR could support the replication of RNA-2 (van Bokhoven et al., 1993). This failure to complement was not due to the transgene inducing resistance, as in plants transgenic for full-length RNA-1, because the plants were fully susceptible to CPMV infection. It is also unlikely to reflect the translatability of the modified RNA-1 because pMB200 was shown to be able to produce significant quantities of the RNA-1-encoded proteins in transient expression studies in protoplasts (van Bokhoven et al., 1993). It probably reflects the fact that, in the absence of replication, only low levels of transgene-derived mRNA accumulate in plants transformed with 1NT-32E. Thus, only a very limited amount of the replicase proteins will be present in the cells. We have also observed that plants transgenic for versions of RNA-1 harboring deletions within the 200K coding region cannot complement RNA-2 (Liu and Lomonossoff, unpublished).

The finding that plants transgenic for a version of RNA-1 lacking the 5′ UTR were not resistant to CPMV, although consistent with the idea that the resistance obtained with full-length RNA-1 is associated with its ability to replicate, contrasts somewhat with the results of Sijen et al. (1995). These authors found that two out of four lines of *N. benthamiana* transgenic for an RNA-1 construct (pBINB200) containing the entire 200K ORF but lacking the 5′ UTR gave rise to some progeny in the R1 generation that were resistant to CPMV infection. This more sporadic resistance is typical of RNA-mediated pathogen-derived resistance (PDR) that does not involve replication of transgene-derived mRNA (Sijen et al., 1996). We have found a similar type of sporadic resistance in plants transgenic for deleted versions of RNA-1 encoding just the carboxy-terminal portion of the 200K protein and the 3′ UTR (Liu and Lomonossoff, unpublished). Though we do not know the reason for the apparent difference in the behavior of our 1NT-32E constructs in giving rise to PDR compared to the equivalent construct of Sijen et al. (1995), it may simply reflect the small number of lines we analyzed or the integration pattern of the transgenes. In this regard, it may be significant that all the RNA-1 transgenic lines of Sijen et al. (1995) that showed resistance had two copies of the transgene.

Based on the ability of CPMV infections to reverse PTGS in silenced plants, Voinnet et al. (1999) suggested that the virus encodes a weak suppressor of PTGS. The ability of plants transformed with RNA-2 to complement exogenously supplied RNA-1, their susceptibility to virus infection, and the ability of RNA-2 to counteract the amplicon effect of RNA-1 when introduced by cotransformation or by crossing suggested that such a suppressor lies on this genome segment. The data obtained from the GFP

patch tests and the PVX experiments confirmed that the CPMV suppressor of PTGS is, indeed, encoded by RNA-2, and that this function is provided by the S coat protein. The suppressor activity identified in these tests is weak compared with that of HC-Pro, a finding consistent with the observations of Voinnet et al. (1999). The identification of a coat protein as a suppressor of PTGS is unusual but not unique: the TCV suppressor was recently identified as its coat protein, P38 (Qu et al., 2003; Thomas et al., 2003). P38 is not thought to have suppressor activity when assembled into particles due to shielding of the interacting domain. Our data do not provide any firm insights as to whether the CPMV suppressor is functional in an assembled or non-assembled form, or in both. During infection, the S subunits are invariably associated with L subunits, even after processing at the L–S junction has occurred. In the absence of such an association, the S protein is unstable in plant cells (Wellink et al., 1996), which probably explains our failure to produce detectable levels of the isolated S subunit in the patch tests. Thus, though the suppressor activity is defined by sequences within the S coat protein, suppression of PTGS is likely to actually be mediated by molecules containing the sequence of both the L and S proteins. The relative weakness of the suppressor might be explained if the suppressor activity of the S coat protein is manifested only before its incorporation into fully assembled viral particles, that is, by some assembly intermediate that accumulates to only low concentration. Alternatively, the suppressor function may act when the S protein is incorporated into particles. In this regard, it is perhaps relevant that one region of the S coat protein that is highly exposed in assembled particles consists of the C-terminal 24 amino acids. It may be significant that deletion of portions of this sequence, though not directly affecting assembly, impairs virus growth (Taylor et al., 1999).

The demonstration that RNA-2 encodes a weak suppressor of PTGS explains why RNA-2 supplied by crossing, but not by exogenous application, can overcome the RNA-1-mediated silencing. In the case of crossing, the RNA-1-mediated silencing will have been reset on meiosis (De Carvalho et al., 1995; Dehio and Schell, 1994); thus, mRNA from the introduced RNA-2 transgene will be able to multiply and express its proteins, including the suppressor function associated with the S coat protein during development. Because the simultaneous presence of a suppressor can prevent the establishment of amplicon-mediated PTGS in PVX (Mallory et al., 2002), the presence of both RNA-1 and RNA-2 in transgenic plants will lead to the establishment of a productive infection. In exogenous application of RNA-2, PTGS has already been established, and the subsequently supplied RNA-2 will neither be replicated nor the polyprotein encoded by it be processed. Thus, no mature S protein, or assembly intermediates containing it, will be produced to counteract the PTGS induced by RNA-1 replication. It should also be noted that though having only weak activity in patch-tests and PVX-based assays, the CPMV suppressor is very effective at preventing RNA-1 acting as an amplicon as long as it is present before silencing is established. As this is the situation, which will actually be encountered during a CPMV infection, the suppressor activity of the S protein appears quite adequate for its biological function.

Materials and methods

Materials

Plasmids pBinPS1NT and pBinPS2NT (Fig. 1A) containing full-length copies of CPMV RNA-1 and RNA-2, respectively, in the binary transformation vector pBINPLUS (van Engelen et al., 1995) have been described previously (Liu and Lomonossoff, 2002). The plasmids were maintained in *Agrobacterium tumefaciens* strain LBA4404. Plasmids based on pgR106 (Jones et al., 1999) were maintained in *A. tumefaciens* strain C58C1 (pCH32). Plasmids 35S-HC-Pro, containing the sequence the HC-Pro suppressor from PVY (Hamilton et al., 2002) and 35S-P38, containing the P38 suppressor from TCV (Thomas et al., 2003) in the vector pBIN61 (Bendahmane et al., 2002) were obtained from D.C. Baulcombe and A.J. Maule, respectively.

Construction of a version of RNA-1 with a deleted 5′ UTR

A version of RNA-1 with a deletion in the 5′ UTR was created using plasmid pCP1, which contains a full-length copy of RNA-1 downstream of a CaMV 35S promoter (Dessens and Lomonossoff, 1993). Oligonucleotide-directed mutagenesis was carried out on double-stranded plasmid DNA to introduce a unique (*Stu*I) restriction site immediately downstream of the 35S promoter sequence and a unique *Cla*I site just upstream of the initiation codon for the 200K protein. Following digestion with *Cla*I and *Stu*I and filling-in with *E. coli* DNA polymerase (Klenow fragment), the plasmid was religated. This resulted in the creation of construct pN81-32E that contains the 35S promoter linked to the sequence of CPMV RNA-1 from nucleotide 205 to its 3′ end, including part of the poly(A) tail. The sequence encoding the deleted version of RNA-1 attached to the 35S promoter was excised by digestion with *Sac*II (which cuts upstream of the 35S promoter) and *Mlu*I (which cuts downstream of the poly(A) tail) and ligated into similarly digested pN81-NT (Liu and Lomonossoff, 2002) to give plasmids pN81-32E, thereby positioning a *nos* terminator downstream of the RNA-1-specific sequence. Finally, the *Asc*I–*Pac*I fragment from pN81-32E was ligated into *Asc*I–*Pac*I-digested pBINPLUS to give pBinP32E (Fig. 1A).

Production of lines of transgenic plants

N. benthamiana was transformed with pBINPLUS-based plasmids using the leaf disk method of Horsch et al. (1985).

Putatively transformed plants were regenerated on medium containing 50 μg/ml kanamycin and finally transferred to soil. Seed from lines of transgenic plants were germinated on plates containing 50 μg/ml kanamycin before planting. The presence of the appropriate transgene was confirmed by PCR analysis on extracted genomic DNA.

Electron microscopy

Tissue samples were fixed samples for 1 h with 1% (w/v) osmium tetroxide in 50 mM sodium cacodylate, pH 7.2, before infiltration with LR White resin, polymerization taking place at 60°C. Ultrathin sections were cut using an Ultracut E microtome (Reichert-Jung) and tissue sections were collected on grids and examined using a JEOL 1200 EX transmission electron microscope.

Inoculation of plants

Agro-infiltration of plants with CPMV constructs was carried out as described by Liu and Lomonossoff (2002). Sap transmission studies were carried out by homogenizing leaf tissue in 10 mM sodium phosphate, pH 7.0, followed by mechanical inoculation of test plants. Agro-infiltration of *N. benthamiana* leaves with constructs 35S-HC-Pro and 35S-P38 was carried out as described by Voinnet et al. (1998).

RNA analysis

RNA was extracted from leaf tissue using the RNeasy Plant Mini Kit (Qiagen, Crawley, UK), fractionated on formaldehyde-containing agarose gels and the RNA transferred to positively charged nylon membranes (Roche, Indianapolis, USA). Strand-specific probes were synthesized from a 1398 bp segment of RNA-1 (corresponding to nucleotides 3030–4428) inserted between the SP6 and T7 promoters of plasmid pCRII-TOPO (Invitrogen Ltd., Paisley, UK) by transcription in vitro with the appropriate RNA polymerase. A probe specific for GFP mRNA was synthesized from plasmid pA949.3, which contains the complete GFP coding sequence inserted into pGEMT-Easy, using T7 RNA polymerase. In all cases, transcription was carried out in the presence of digoxigenin (Roche) and the bound probe was detected by chemiluminescence.

Creation of constructs containing portions of the RNA-2 polyprotein

The region of CPMV RNA-2 encoding the various proteins was PCR amplified from pBinPS2NT using appropriate primers. As the mature CPMV proteins are generated in planta by proteolytic cleavage of precursors, start and stop codons were engineered where necessary to obtain correct expression. Thus, the sequences encoding the 58K, 48K, and L coat proteins had a termination codon inserted following that encoding the C-terminal amino acid. The sequence encoding the S coat protein had an initiation codon inserted before the natural N-terminal glycine.

In the case of sequences to be inserted into pBIN61, the PCR primers were designed so that an *Xba*I site would be present at the 5′ end of the coding sequence and either a *Bcl*I (58/48K and 48K) or a *Bam*HI (L, S, and L–S) site at its 3′ terminus. Following digestion with *Xba*I and either *Bcl*I or *Bam*HI, the coding sequences were ligated into *Xba*I/*Bam*HI-digested pBIN61. For insertion into the PVX vector, the PCR primers incorporated *Cla*I and a *Sal*I sites at the 5′ and 3′ termini of the product, respectively. The sequences were inserted between the *Cla*I and *Sal*I sites of the PVX vector pgR106 (Jones et al., 1999) allowing them to be expressed from the duplicated PVX coat protein subgenomic promoter during PVX infection (Fig. 1B).

GFP patch tests on N. benthamiana

Leaves of 4-week-old nontransgenic *N. benthamiana* and line 16c (Ruiz et al., 1998) were inoculated as described by Voinnet et al. (1998) with *A. tumefaciens* strain C58C1 (pCH32) containing the binary Ti vector pBIN61-based constructs. The bacteria were grown to stationary phase. Co-inoculation of two cultures was done by mixing at a 50:50 ratio before inoculation. Co-inoculation of three cultures was done at a 50:25:25 ratio, the strain containing pBIN61-GFP having 50% of the final culture volume.

Silencing and PVX-infection of N. benthamiana plants by agro-inoculation

Leaves of 16c plants were silenced as described by Voinnet et al. (1999) by agro-infiltration with *A. tumefaciens* strain C58C1 containing a binary Ti vector that carried a 35S-inverted-repeat GFP cassette, 35S-GFP-RNAi. At approximately 21 days post-inoculation (dpi), plants exhibiting complete silencing of GFP, which appeared completely red under ultraviolet (UV) light, were either infected with CPMV or were agro-infiltrated with C58C1 cells containing the PVX derivatives shown in Fig. 1B. The silencing status of leaves of plants exhibiting symptoms of PVX infection was subsequently examined under UV light at approximately 14–21 dpi. To confirm the genetic stability of the PVX constructs, RNA was extracted from leaves exhibiting symptoms of PVX infection and used as template for RT-PCR, using the oligonucleotides CACTTAGAATTCGAAGTAAATACATATCTC and TGTGGCAGGAGTTGCGCC as primers to amplify the region including the PVX 8K coding sequence and the CPMV-specific sequences.

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